Supporting Information

Heat, pH, and salt: synthesis strategies to favor formation of near-infrared emissive DNA-stabilized silver nanoclusters

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1 Experimental details

1.1 General Methods and Materials.

Reagents used include DNA (Integrated DNA technologies, standard desalting), ammonium acetate (99.8%, Fisher chemicals), silver nitrate (≥99.9999%, Sigma Aldrich), and sodium borohydride (99.99%, Sigma Aldrich). Samples were prepared in ultrapure MilliQ water.

Table S1. Optimized synthesis conditions for Ag_N-DNAs studied here. λ_p denotes the peak emission wavelengths for the Ag_N-DNA products formed using each DNA template sequence.

Name	DNA sequence 5' to 3'	λ_p / nm	[DNA]/ μM	[$AgNO_3$]/ μM	Optimized storage temperature
DNA-**1**	ACCGCCGGGA	680/820	25	125	21 °C after heating at 37 °C for 4 h
DNA-**2**	CGAACCGGGC	644/820	25	187.5	21 °C after heating at 37 °C for 4 h
DNA-**3**	AGGCGATCAT	580/844	25	187.5	21 °C after heating at 37 °C for 4 h
DNA-**4**	GCCCCCCCGC	670	25	125	21 °C after heating at 37 °C for 4 h
DNA-**5**	CACCCCGAGC	714	20	300	21 °C
DNA-**6**	TAGCCCCTGT	562	25	125	4 °C

1.2 Comparison of synthesis procedures of AgN-DNAs between high-throughput experiments and single microcentrifuge tubes.

When Ag_N-DNAs are prepared in single microcentrifuge tubes (solution volumes vary from ~100 μL to 1.5 mL), a solution of DNA oligomers and Ag^+ ions is first mixed by vortexing, then held at room temperature, and finally chemically reduced 15 minutes later by addition of $NaBH_4$ and vortex mixing. Solutions are then immediately stored at 4 °C in the dark until measurement, which is often performed periodically over a few days to assess chemical yield. When Ag_N-DNAs are prepared in high-throughput, an automated liquid handling robot is used to synthesize Ag_N-

DNAs in a 384 microwell plate, with 40 μL per sample. First, a solution of Ag^+ and DNA is mixed by up to 10 pipetting cycles. 18 minutes later, the solution is reduced by the addition of $NaBH_4$ and a second round of pipet mixing. Pipets are thoroughly washed between steps with ultrapure water to avoid any contamination. The entire process takes place at room temperature. One day after high-throughput synthesis, UV-excited visible emission spectra are collected to verify proper chemical reduction, a process that takes approximately 4 hours to complete on a commercial well plate reader.[1-41-4] Emission spectra are again collected one week after Ag_N-DNA synthesis, and the spectral properties at one week are reported in the literature, as these represent more time-stable products.

1.3 Optimization of storage temperature

At first, a stoichiometric amount of $AgNO_3$ (**Table S1**) was added to the single-stranded DNA oligomer in 10 mM NH_4OAc (pH 7.0) to form Ag^+–DNA complexes. After 15 minutes, a freshly prepared aqueous solution of $NaBH_4$ ($[BH_4^-]/[Ag^+] = 0.5$) was added to the Ag^+–DNA solution. The sample was divided into four samples of equal volume. The first sample was stored at 4 ºC in the dark, the second sample was stored at room temperature in the dark, and the remaining two samples were heated at 37 ºC for 4 hours while protected from light exposure. After heating, one of the samples was stored at 4 ºC and the other at room temperature in the dark. The Ag_N-DNAs were allowed to form over two days under the conditions mentioned above. After 2 days, the samples were transferred to a microwell plate, and the emission spectra were collected using a Tecan Spark plate reader with 260 nm excitation (SI Section 2.1).

1.4 pH tests

20 mM NH_4OAc stock solutions were prepared at pH 4, 5, 6, 7, 8, 9, and 10. To do so, the pH of the 20 mM NH_4OAc solution was adjusted by the addition of acetic acid and sodium hydroxide. These 20 mM NH_4OAc stock solutions were diluted to 10 mM NH_4OAc to synthesize the Ag_N-DNAs. The Ag_N-DNAs were synthesized using the optimized DNA and silver nitrate concentrations in 10 mM NH_4OAc solution mentioned in section **1.2** and **Table S1** and then were stored at 4 ºC in the dark for two days. To account for whether the dilution changes the pH of the NH_4OAc solution, in a separate experiment the pH of the NH_4OAc before and after dilutions were recorded. No change in the pH of the NH_4OAc upon dilutions was observed.

1.5 Stability in NaCl solution

Ag_N-DNAs were synthesized using optimized DNA and silver nitrate concentrations in 10 mM NH_4OAc solution at pH 7 and stored at their optimized storage temperature (see **Table S1**). The Ag_N-DNAs were allowed to form for 2 days. Then 20 µL of different concentrations of NaCl stock solutions were added to 20 µL Ag_N-DNAs to achieve the final concentrations of 250 µM to 1000 µM of NaCl. To monitor the changes in intensity with time, the emission intensity of the 20 µL of Ag_N-DNA with 20 mM of added NH_4OAc was used as a control (denoted as 0 µM NaCl). The emission spectra were recorded just after the addition of NaCl (0 h) to 6 h with an interval of 1 h, then after 24 h and 72 h. Due to the dilution with NaCl solutions, the final concentration of Ag_N-DNAs was half the concentration used for heating and pH experiments as mentioned in sections **1.2**, **1.3**, and **Table S1**.

1.6 Calculation of percent intensity change.

$$\% \text{ intensity change} = [(I_{NaCl} - I_0) / I_0] \times 100 \quad \textbf{(1)}$$

where, I_{NaCl} = emission intensity in the presence of different concentrations of NaCl ([NaCl] = 0, 250, 500, 750, and 1000 μM) and I_0 = emission intensity in the absence of NaCl (*i.e*. [NaCl] = 0 μM) at a specific time for a specific λ_p for each Ag_N-DNAs.

All emission spectra of Ag_N-DNAs in the presence of NaCl solution (0 μM to 1000 μM) at different intervals of time are shown in **Figures S2** to **S6**. These emission intensities were used to calculate the % change in intensities reported in **Figures 3** and **4**. For nanoclusters with two distinct species, one red or far-red and the other NIR (DNA-**1**, **2**, and **3**), results are shown for the NIR species emission changes with time and NaCl concentrations in **Figure S7**. We found that for NIR wavelengths, emission intensity tends to decrease with time after the addition of NaCl, indicating that the nanoclusters are not stable in the presence of NaCl.

Table S2. Summary of effects of storage temperature, pH, and the presence of NaCl on the formation of Ag_N-DNAs.

Name	**Storage conditions for the highest chemical yield**	**pH**	**Behavior in the presence of NaCl at specific λ_p / nm**	
DNA-**1**	37 ºC for 4 h then at 21 ºC	7	680 nm: stable and increases at 750 and 1000 μM	820 nm: Stable at 750 μM only
DNA-**2**	37 ºC for 4 h then at 21 ºC	7 and 9	644 nm: Stable and increases	846 nm: Unstable
DNA-**3**	37 ºC for 4 h then at 21 ºC	9	580 nm: Unstable	844 nm: Unstable
DNA-**4**	37 ºC for 4 h then at 21 ºC	7	670 nm: Stable and increases	
DNA-**5**	21 ºC	10	714 nm: Stable, increases and remains constant	
DNA-**6**	4 ºC	6	562 nm: Stable and increases	

2 Optical characterization

2.1 UV-Vis and fluorescence spectroscopy.

Emission spectra of synthesized Ag_N-DNAs were collected in a Tecan Spark instrument in a 400 nm to 850 nm range. The Ag_N-DNAs were universally excited at 260 nm[5] with an excitation bandwidth of 20 nm and an emission bandwidth of 10 nm with a gain manual set at 75, or at 60 when the emission intensity saturates the detector with a gain set at 75. The full emission spectra of NIR Ag_N-DNAs (λ_p > 700 nm) were collected using a thermoelectrically cooled, fiber-coupled spectrometer (Ocean Optics QE65000). Absorbance spectra were collected using a DH-Mini (Ocean Insight) deuterium and tungsten halogen UV–vis–NIR light source. Fluorescence spectra were collected using a UV LED as a light source for universal UV-excitation of the nanoclusters.[5] The full spectra of Ag_N-DNAs are shown in **Figure S1**. The emission spectra of Ag_N-DNAs in the presence of different concentrations of NaCl solution were recorded at different time intervals and are shown in **Figures S2 – S6**.

2.1.1 Emission and absorbance spectra of Ag_N-DNAs reported in this study.

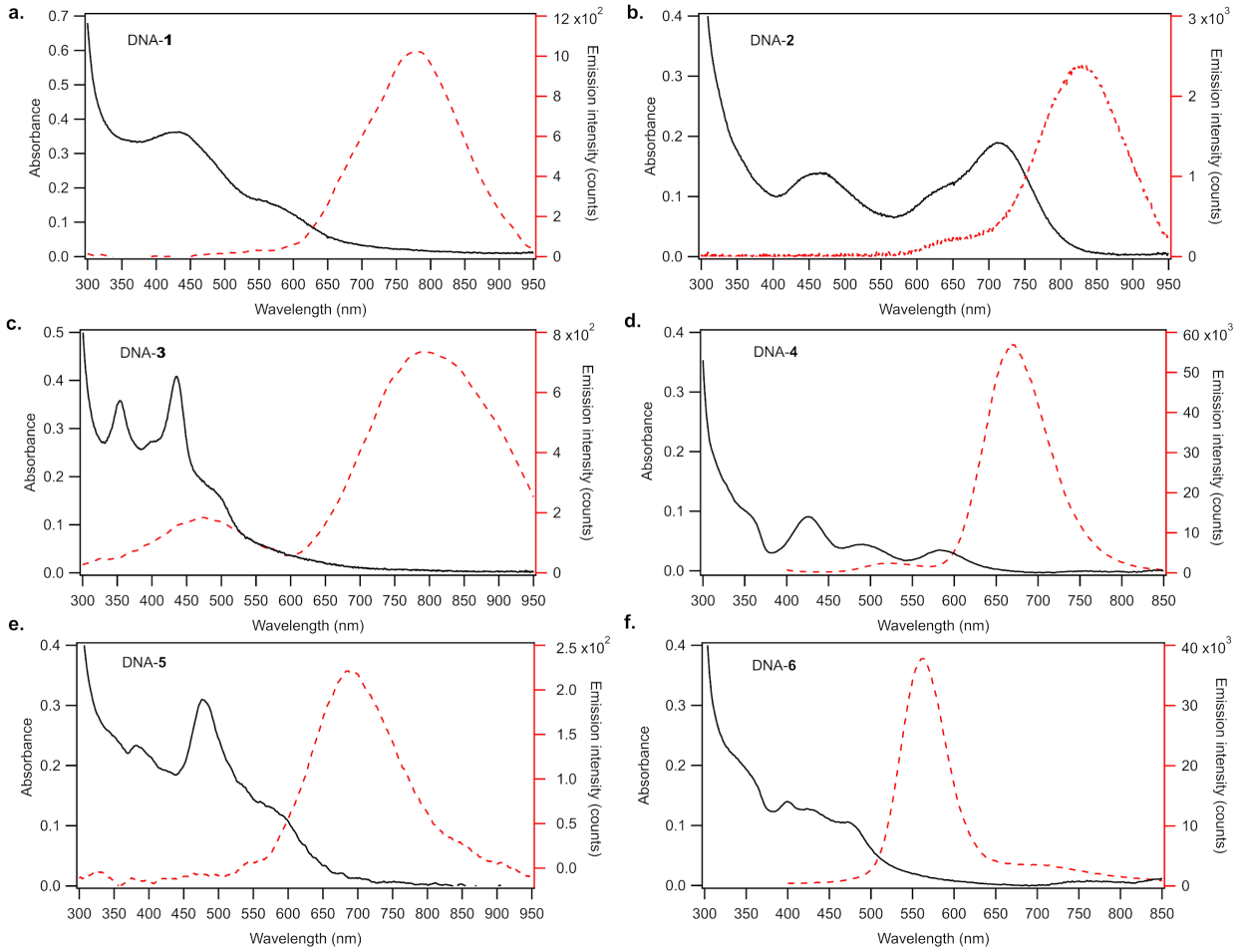

Figure S1. Absorbance (black solid) and emission spectra (red dashed) of Ag_N-DNAs studied in this work. The Ag_N-DNAs were synthesized at the optimized storage temperature and at pH 7, as mentioned in **Table S1**.

2.1.2 Emission spectra of Ag_N-DNAs in the presence of different concentrations of NaCl solution.

1. DNA-**1**

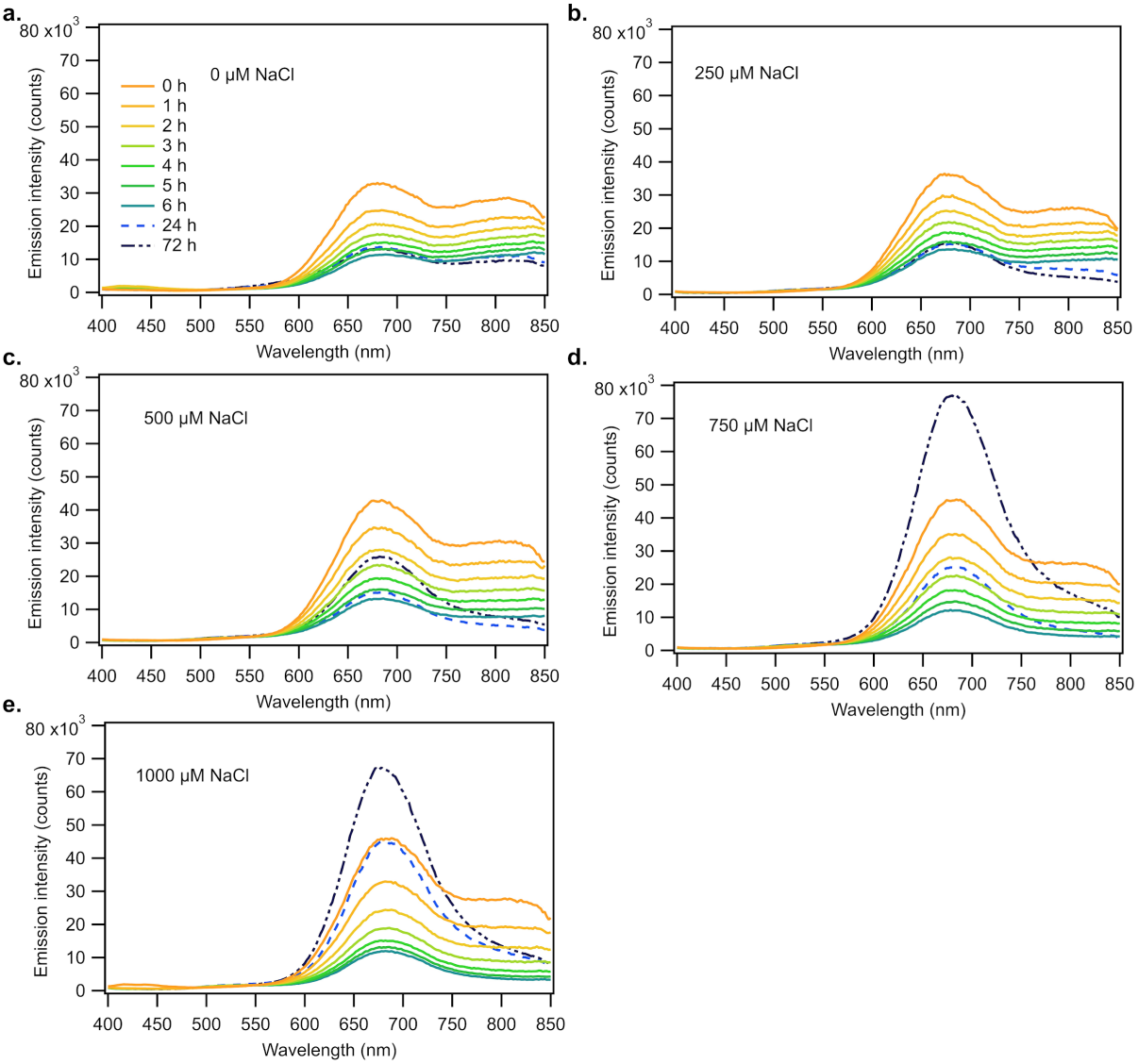

Figure S2. Emission spectra of Ag_N formed from DNA-**1** in the presence of **a)** 0 µM, **b)** 250 µM, **c)** 500 µM, **d)** 750 µM, and **e)** 1000 µM of NaCl measured at different intervals of time.

2. DNA-**2**

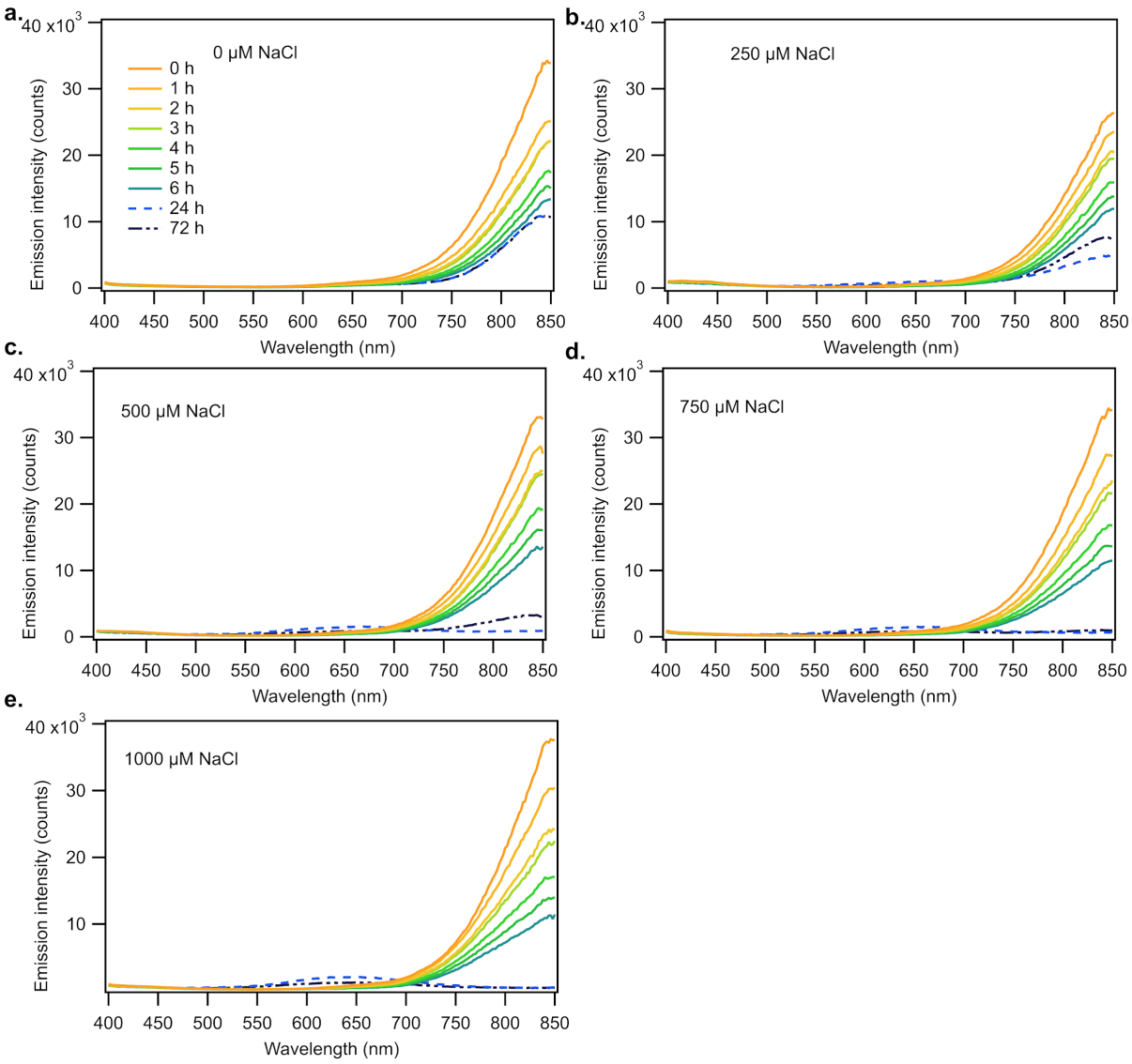

Figure S3. Emission spectra of Ag_N formed from DNA-**2** in the presence of **a)** 0 µM, **b)** 250 µM, **c)** 500 µM, **d)** 750 µM, and **e)** 1000 µM of NaCl measured at different intervals of time.

3. DNA-**3**

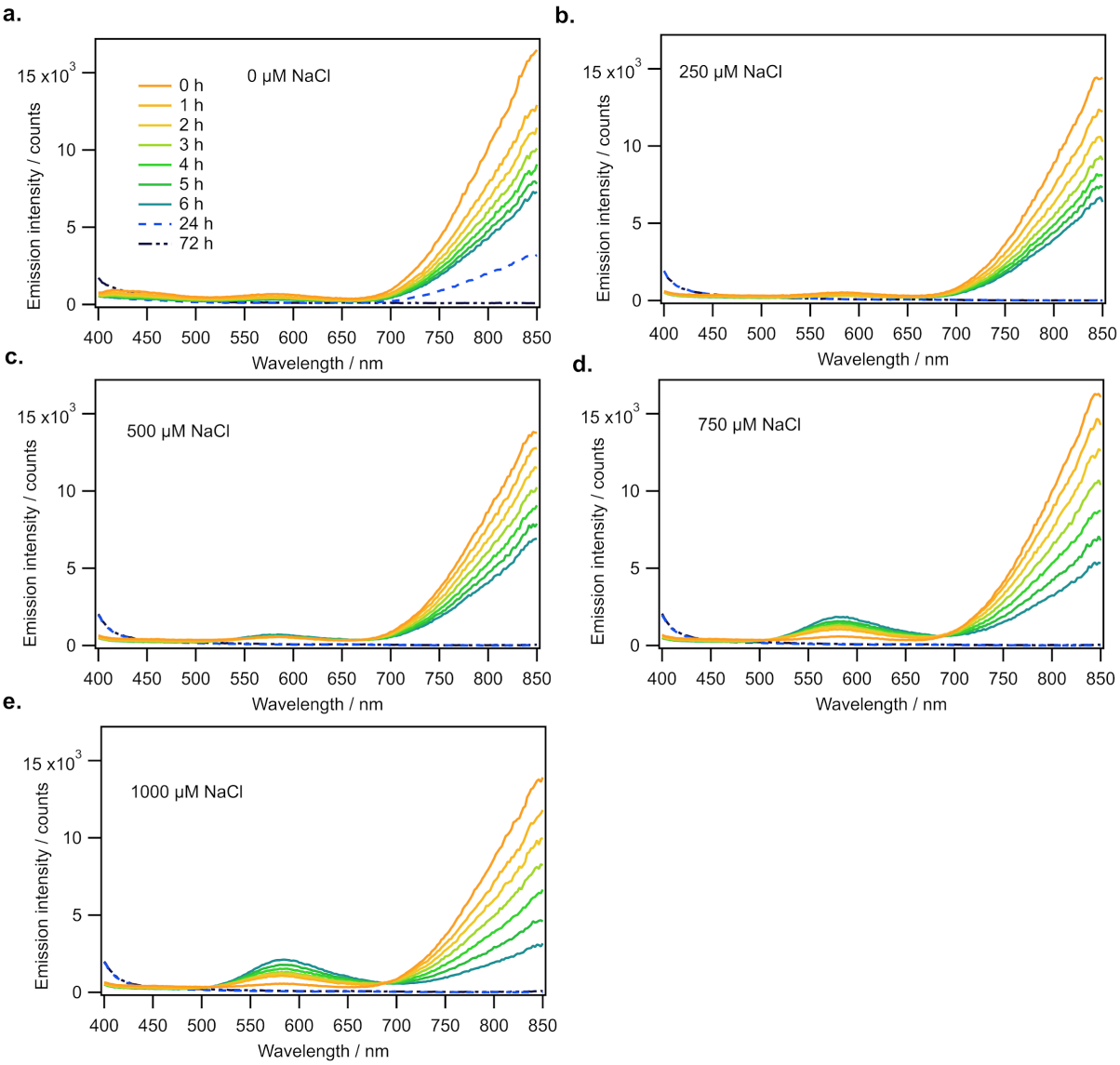

Figure S4. Emission spectra of Ag_N formed from DNA-**3** in the presence of **a)** 0 µM, **b)** 250 µM, **c)** 500 µM, **d)** 750 µM, and **e)** 1000 µM of NaCl measured at different intervals of time.

4. DNA-**4**

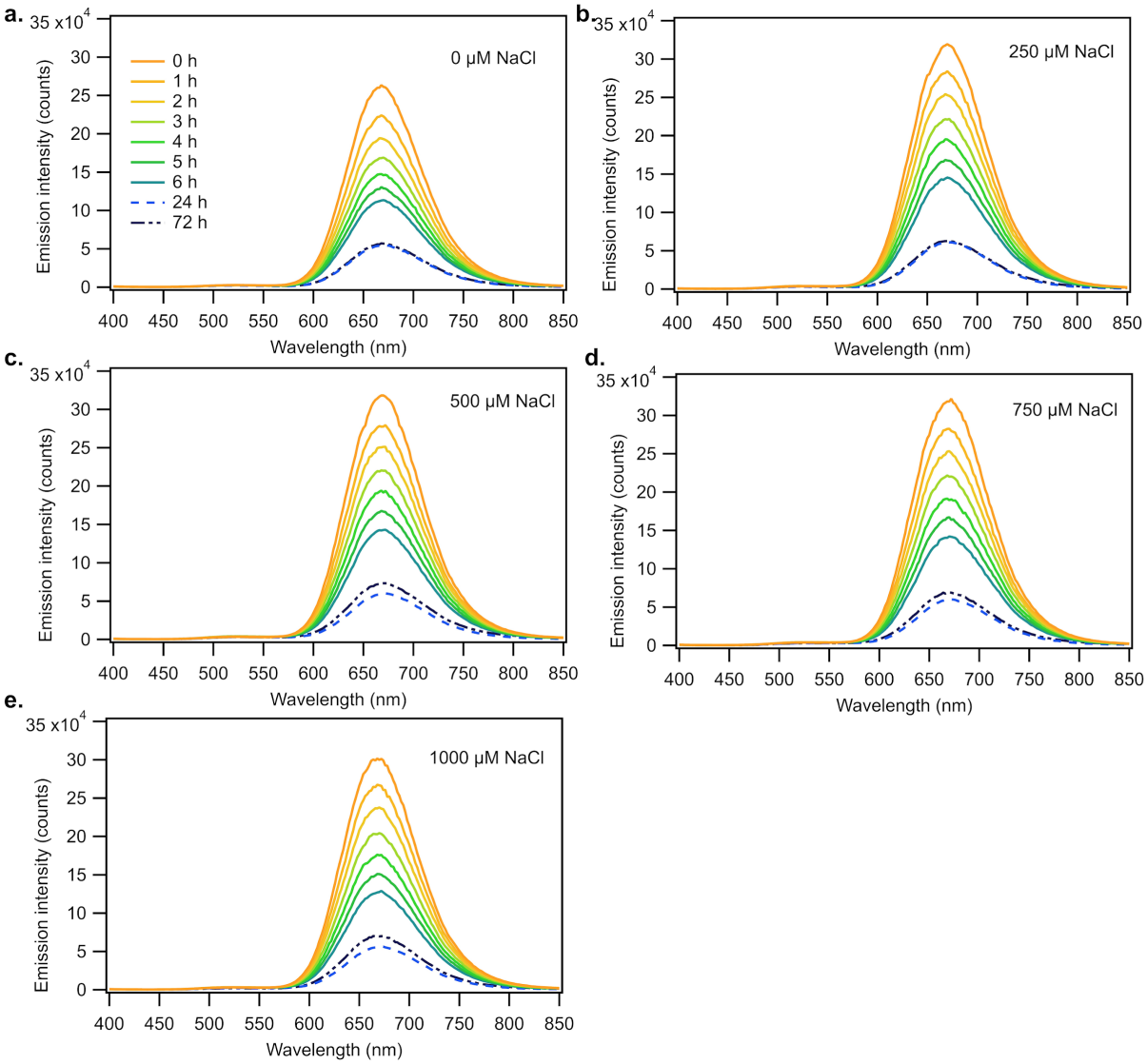

Figure S5. Emission spectra of Ag_N formed from DNA-**4** in the presence of **a)** 0 μM, **b)** 250 μM, **c)** 500 μM, **d)** 750 μM, and **e)** 1000 μM of NaCl measured at different intervals of time.

5. DNA-**5**

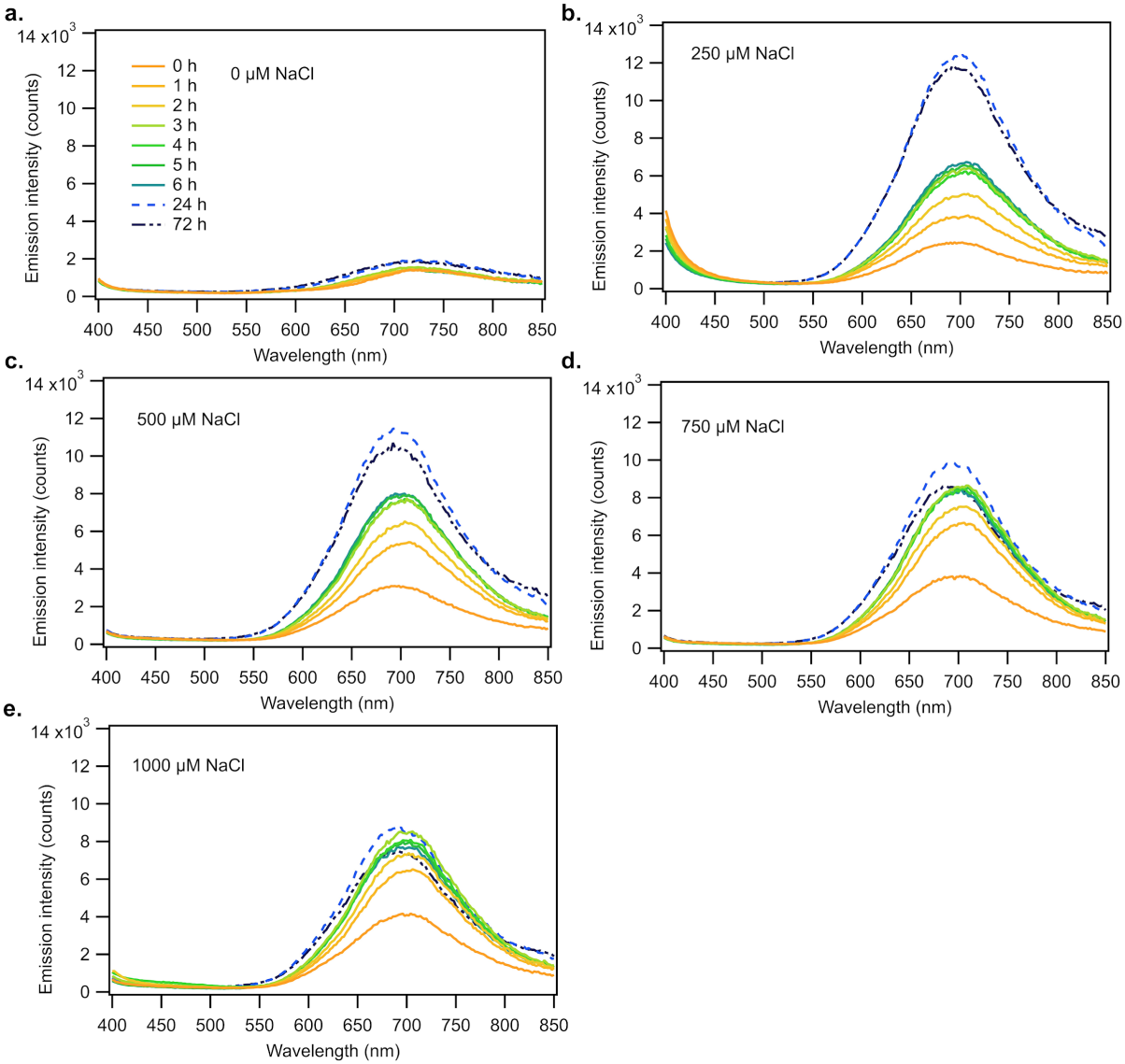

Figure S6. Emission spectra of Ag_N formed from DNA-**5** in the presence of **a)** 0 μM, **b)** 250 μM, **c)** 500 μM, **d)** 750 μM, and **e)** 1000 μM of NaCl measured at different intervals of time.

2.1.3 Percent intensity change of NIR-emissive species of Ag_N-DNAs in the presence of different concentrations of NaCl solution.

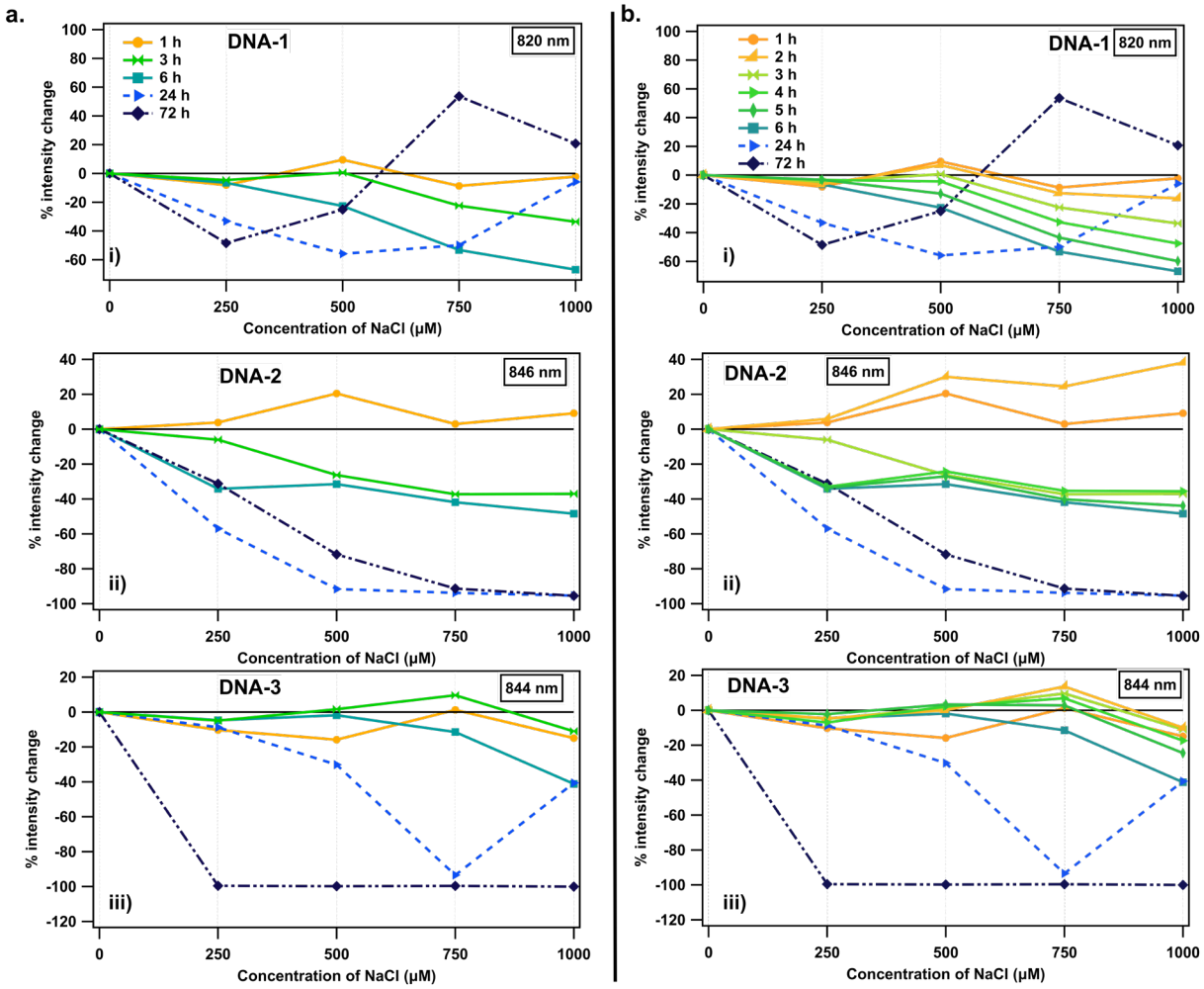

Figure S7. a. Percent intensity change for NIR emissive-Ag_N synthesized from **i)** DNA-**1, ii)** DNA-**2, iii)** DNA-**3**. Each graph in **a**. compares the change in emission intensity after the addition of each concentration of NaCl, 0 μM to 1000 μM, at 1 h (yellow with circle), 3 h (green with double triangles), 6 h (teal with squares), 24 h (bright blue with triangles), and 72 h (navy with diamonds) at a specific wavelength. **b.** Percent intensity change for NIR emissive-Ag_N synthesized from **i)** DNA-**1, ii)** DNA-**2, iii)** DNA-**3**. Each graph in **b**. compares the change in emission intensity after the addition of each concentration of NaCl, 0 μM to 1000 μM, at 1, 2, 3, 4, 5, 6, 24, and 72 h at a specific wavelength.

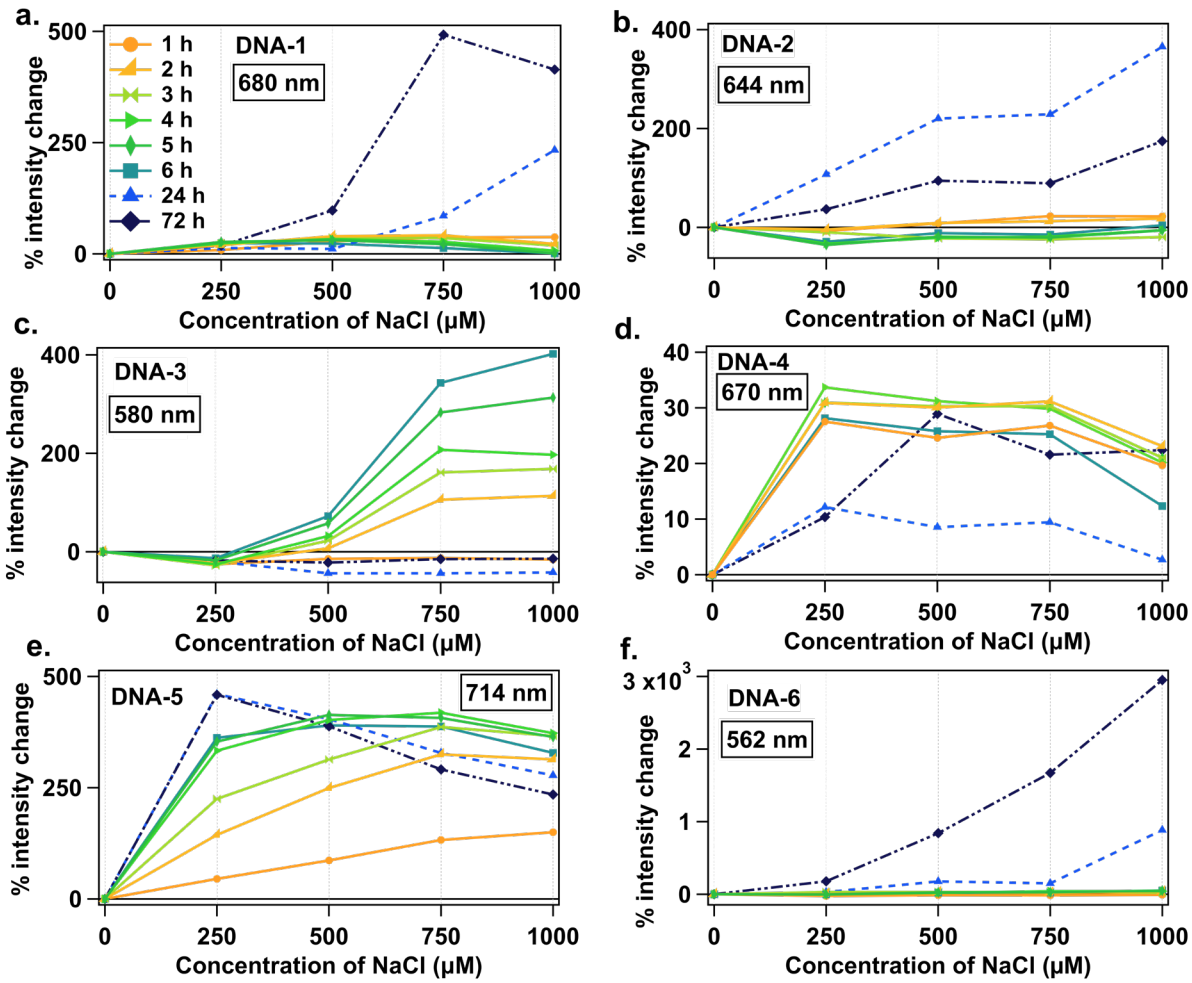

Figure S8. Percent intensity change for Ag_N synthesized from **a)** DNA-**1**, **b)** DNA-**2**, **c)** DNA-**3**, **d)** DNA-**4**, **e)** DNA-**5**, and **f)** DNA-**6**. Each graph compares the change in emission intensity after the addition of each concentration of NaCl, 0 μM to 1000 μM, at 1, 2, 3, 4 , 5 , 6, 24, and 72 h at a specific wavelength.

3 HPLC purification of Ag_N-DNAs.

The HPLC method generally involves a 5 min pre-injection at 95% of the aqueous solvent that contains 35 mM TEAA in H_2O (**Solvent A**) and 5% of 35 mM TEAA in MeOH (**Solvent B**). Elution steps are as follows: 0 – 2 min: 95% to **x**% solvent A, 2 – 12 min: **x**% – **y**% of solvent A, 12 – 14 min: **y**% – 5 % of solvent A, 14 – 19 min: 95% of solvent A. The flow rate and the gradient (**x**% to **y**% of solvent A in 10 minutes, **Table S2**) used for purification are optimized for each Ag_N-DNA to ensure maximum separation from synthesis byproducts. Collection of pure fractions of Ag_N-DNAs is based on the absorbance (DAD) and emission (FLD) signals collected for the absorbance of the DNA at 260 nm (red), absorbance peak specific to Ag_N-DNA (yellow), and emission peak of the emitter by universally exciting all Ag_N-DNAs at 260 nm (blue).[5] The time of aliquot collection is in **Table S2** and shown in the grey-shaded box in HPLC chromatograms (**Figure S9** to **S10**). The HPLC chromatogram of DNA**-3** was previously reported.[6]

Table S2. Experimental conditions used for HPLC purification. (NOTE: Solvent A is 35 mM TEAA in H_2O and solvent B is 35 mM TEAA in methanol).

Name	HPLC Gradient x% to y% of solvent A	Retention time / min
DNA-4	85% to 75% 1.0 mL/min	3.5 – 3.8
DNA-6	90% to 70% 1.0 mL/min	9.8 – 10.5

3.1 HPLC chromatograms.

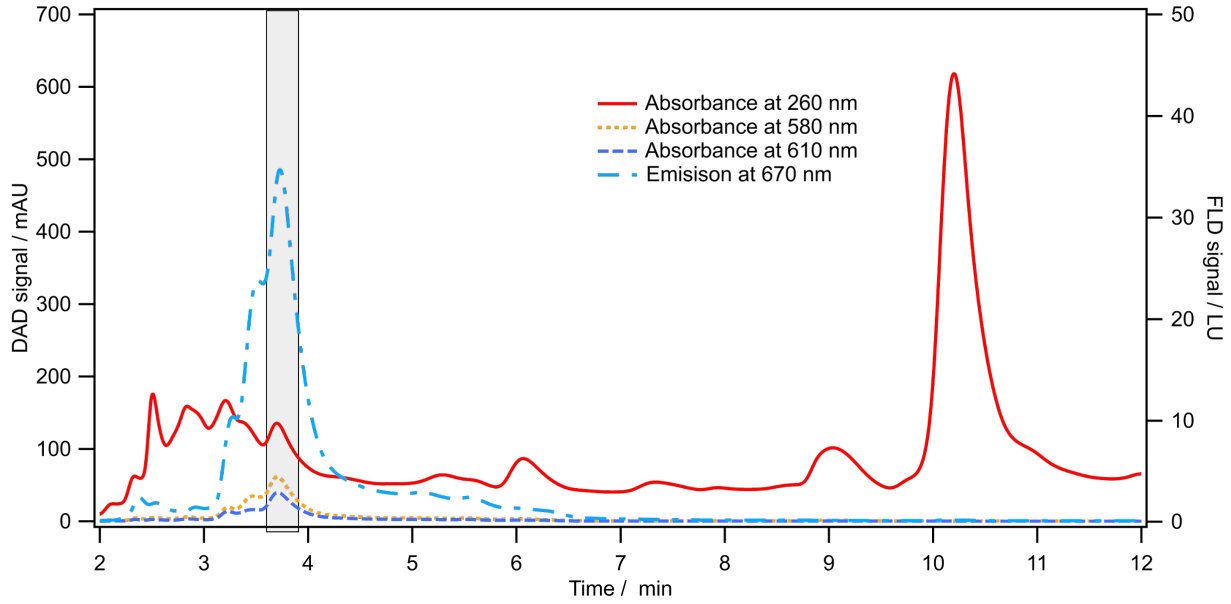

Figure S9. HPLC chromatogram of DNA-**4**. The gray shaded box indicates the fraction collected and used for molecular composition determination. (NOTE: This Ag_N-DNA requires two times HPLC purification using the same solvent gradient to obtain a pure product. The mass spectrum shown in **Figure S12** was obtained after two HPLC purifications of the same sample.)

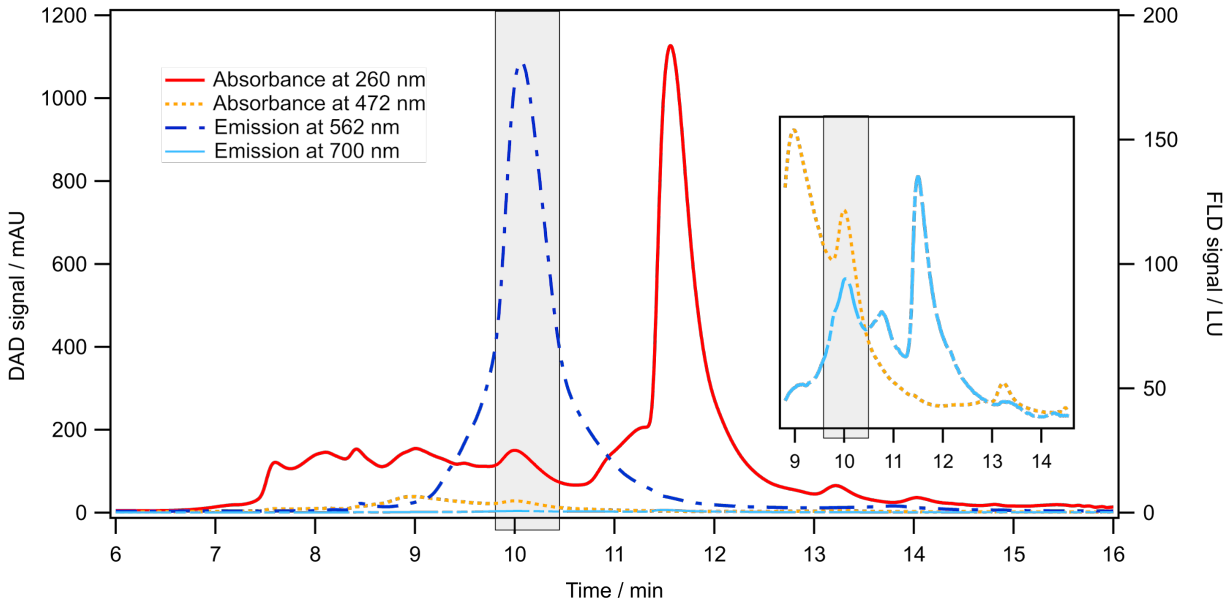

Figure S10. HPLC chromatogram of DNA-**6**. The gray shaded box indicates the fraction collected and used for molecular composition determination. Inset shows zoomed in chromatogram to better show the absorbance chromatogram at 472 nm and emission chromatogram at 700 nm.

4 Comparison of absorbance and emission spectra of Ag_N-DNAs before and after HPLC purification

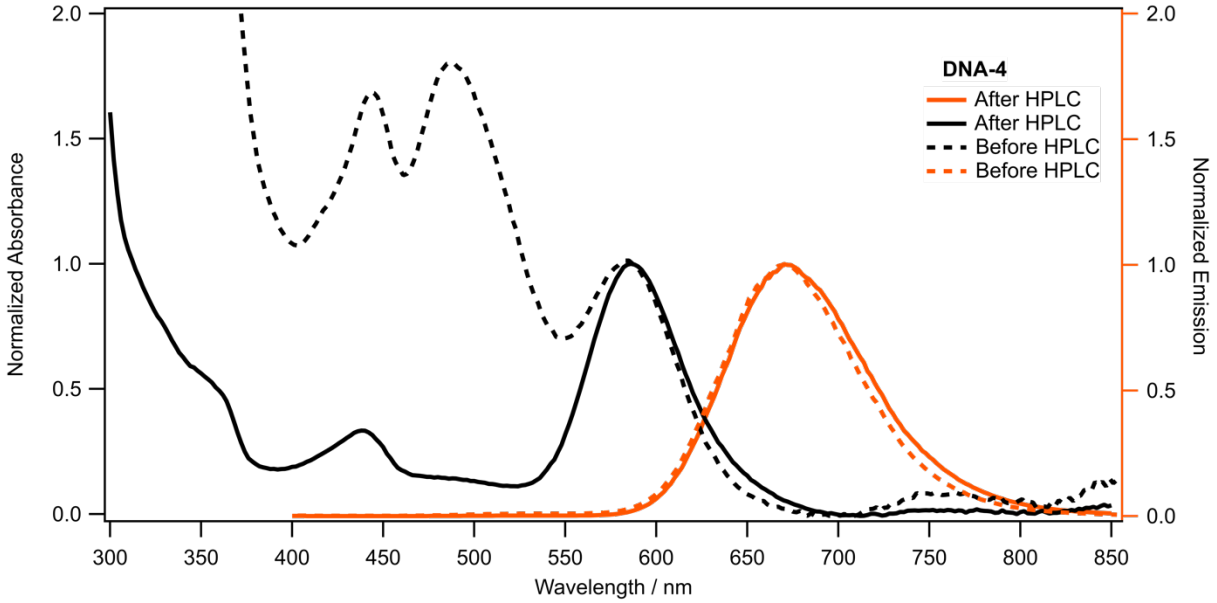

Figure S11. Absorbance (black) and emission (orange) spectra of DNA-**4** measured before (dashed line) and after (solid line) HPLC purification.

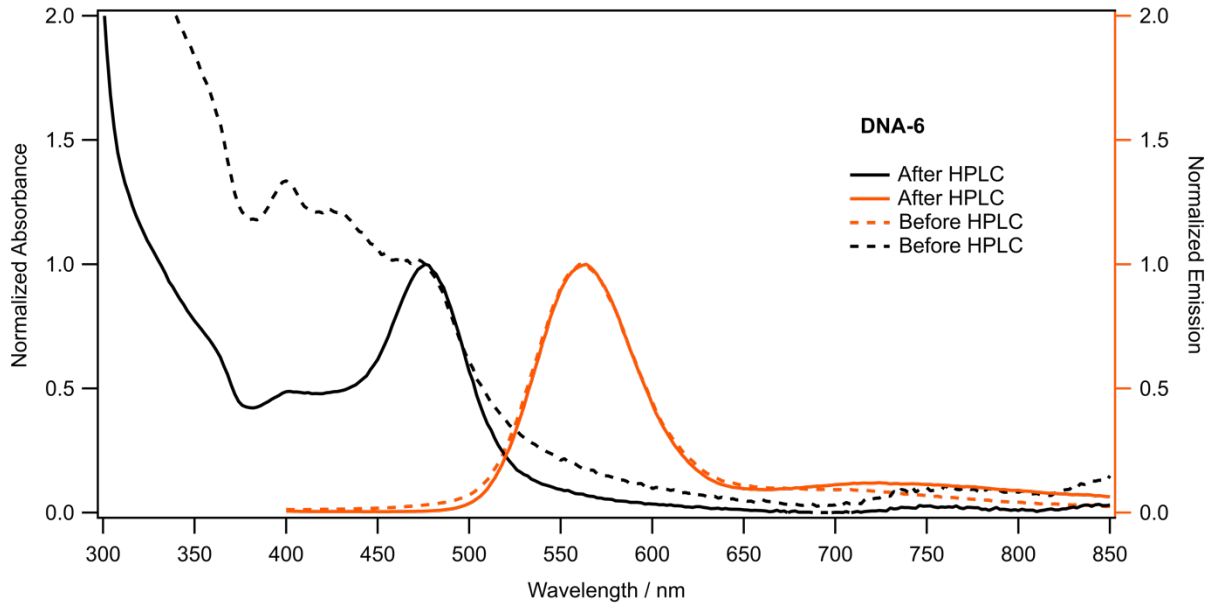

Figure S12. Absorbance (black) and emission (orange) spectra of DNA-**6** measured before (dashed line) and after (solid line) HPLC purification.

It was not possible to separate a single emissive product in the case of DNA-**6**. (Separation of single-emissive species from a heterogeneous solution of Ag_N-DNA formed during the chemical

synthesis is challenging and it is often impossible to obtain a single-emissive product.) Emission spectra of HPLC-purified DNA-**6** showed the presence of two emitters with λ_p = 562 nm and 750 nm still present even after the HPLC purification (**Figure S12**). The fraction collected at *ca.* 10 minutes during the HPLC run was injected into the mass spectrometer, and two Ag_N-DNA species with N_0 = 4 and 6 were identified (see section 5: **Figure S14** and **Table S3**). We hypothesize that the species containing N = 15 and N_0 = 6 is the emitter with λ_p = 750 nm, while the species containing N = 12 and N_0 = 4 is the emitter with λ_p = 562 nm.

5 Mass spectrometry of Ag_N-DNAs

HPLC-purified Ag_N-DNAs were solvent exchanged into 10 mM ammonium acetate and were directly injected at 0.1 mL min^{-1} in negative ion mode with a 2 kV capillary voltage, 30 V cone voltage and no collision energy on a Waters Xevo G2-XS QTof. Spectra were collected from 1000 to 4000 m/z and integrated for 1 s. Source and desolvation temperatures were 80 ºC and 150 ºC, respectively. Gas flows were 45 L h^{-1} for the cone, and 450 L h^{-1} for the desolvation. Samples were injected with 50 mM NH_4OAc – MeOH (80:20) solution at pH 7.

Determination of nanocluster size (total number of silvers N, the number of DNA strands, n_s) and the overall charge, Q_c (and hence the number of effective valence electrons, N_0) was done by fitting the calculated isotopic distribution of the Ag_N-DNA to the experimental spectra (**Figures S12 - S13**). The detailed explanation and formulae used for the calculation of N, N_0, and Q_c have been reported previously.[6-8] Calculated isotopic distributions were obtained from MassLynx using the chemical formula and corrected for the overall positive charge (oxidation state, Q_c) of the nanocluster core (**Table S3**). Mass spectra and isotopic distribution table of DNA-**3** are previously reported in ref. 6.[6] Notations used are summarized below:

n_s = Number of DNA strands

N = Total number of silver atoms

N_0 = The number of effective valence electrons

Q_c = Oxidation state of the nanocluster

$N_0 = N - Q_c$

5.1 Mass spectra of Ag_N-DNAs.

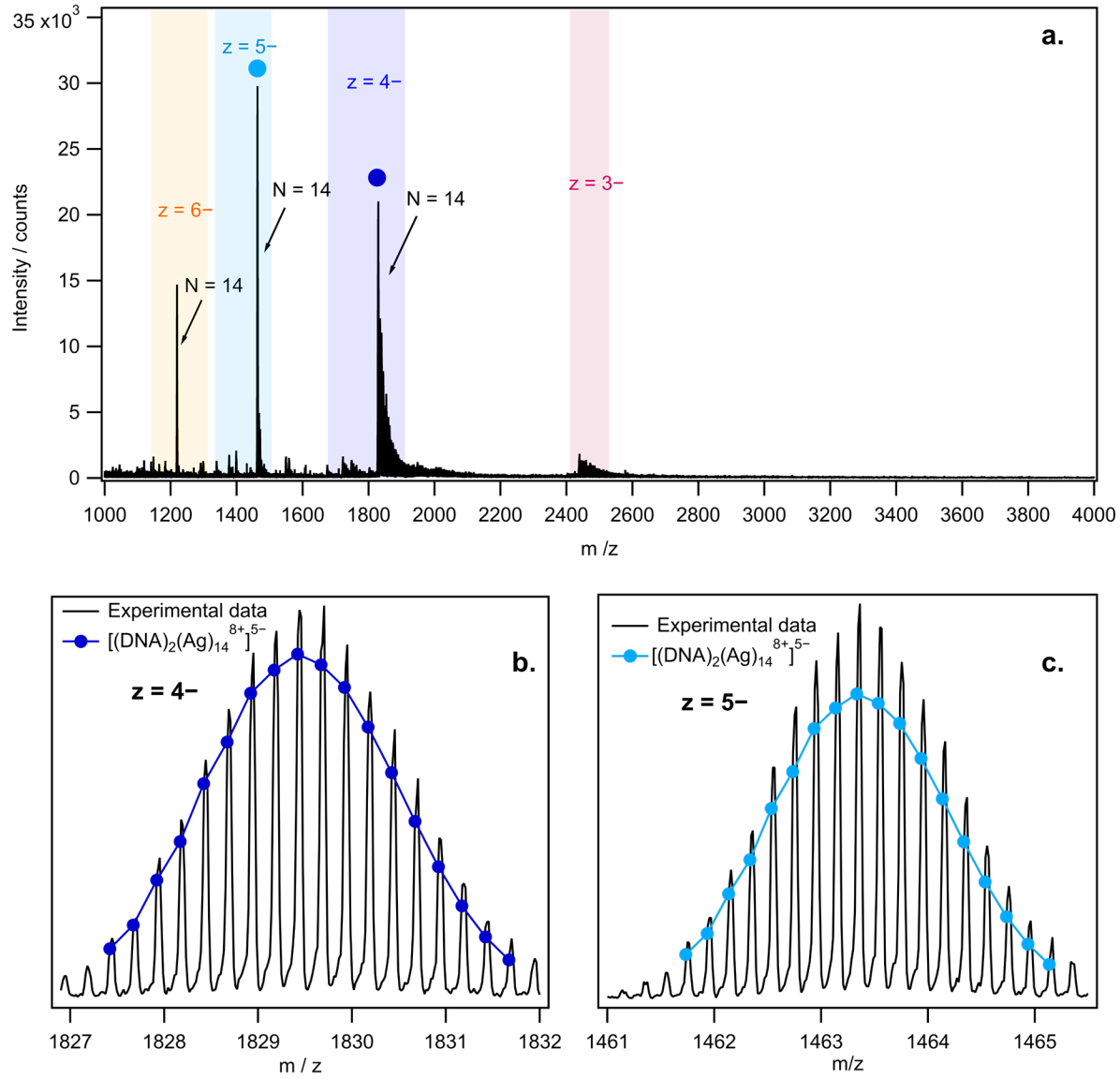

Figure S13. a. Mass spectrum of DNA-**4** (in black) after HPLC purification. Charge states z = 3−, 4−, 5−, and 6− are shaded with different colors. **b, c.** Calculated isotopic distributions for $(DNA)_2[(Ag)_{14}]^{8+}$ at z = 4− (bright blue circles) and z = 5− (light blue circles), respectively. Isotopic distributions were calculated using the chemical formula $C_{184}H_{242}N_{68}O_{116}P_{18}Ag_{14}$. Gaussian fits to experimental and calculated mass spectral peaks are used to assign nanocluster molecular formula (**Table S3**).

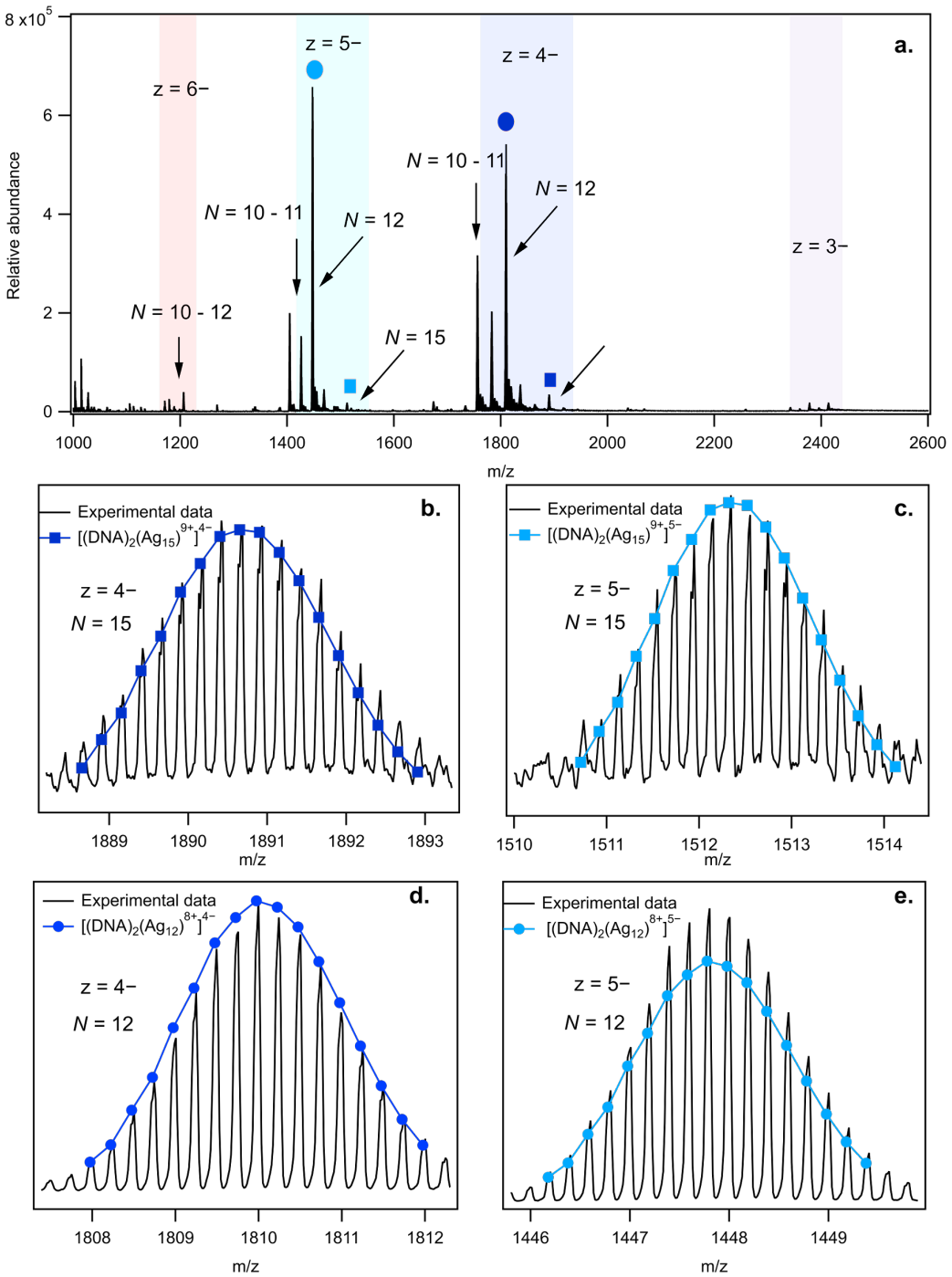

Figure S14. a. Mass spectra of DNA-**6** (black) after HPLC purification. Charge states z = 3−, 4−, 5−, and 6− are shaded with different colors. **b., c.** Calculated isotopic distributions for $(DNA)_2[(Ag)_{15}]^{9+}$ at z = 4− (bright blue squares) and z = 5− (light blue squares) using the chemical formula $C_{192}H_{248}N_{66}O_{120}P_{18}Ag_{15}$. **d.** and **e.** Calculated isotopic distributions for $(DNA)_2[(Ag)_{12}]^{8+}$ at z = 4− (bright blue circles) and z = 5− (light blue circles) using the chemical formula $C_{192}H_{248}N_{66}O_{120}P_{18}Ag_{12}$.

Table S3. Mass spectral analysis to determine molecular formula. Summary of center of Gaussian fits, x_0, to the dominant peaks in the experimentally measured mass spectra of Ag_N-DNAs (**Figure S13** to **S14**) to the calculated mass distributions for different overall nanocluster charges Q_c. The rightmost column compares x_0 for the experimental mass spectral peak to x_0 for the calculated mass distribution at the specific Q_c. Bold text corresponds to experimental values and the corresponding best matching Q_c.

Ag_N-DNA	**Charge state**	**Fit**	**x_0**	**Error**	**Experimental x_0 − Calculated x_0**
DNA-**4**: n_s = 2, ***N*** **= 14**, N_0 **= 6**, Q_c **= 8**, Chemical Formula: $C_{184}H_{242}N_{68}O_{116}P_{18}Ag_{14}$					
DNA-4		*Experimental*	**1829.5**	**0.105**	
	z = 4−	Q_c = 0	1831.5	0.00509	–2.0
	N **= 14**	Q_c = 7	1829.7	0.00509	–0.2
	N_0 **= 6**	Q_c **= 8**	**1829.5**	**0.00509**	**0.0**
		Q_c = 9	1829.2	0.00509	0.3
		Experimental	**1463.4**	**0.0862**	
	z = 5−	Q_c = 0	1465	0.00407	–1.6
	N **= 14**	Q_c = 7	1463.6	0.00407	–0.2
	N_0 **= 6**	Q_c **= 8**	**1463.4**	**0.00407**	**0.0**
		Q_c = 9	1463.2	0.00407	0.2
DNA-**6**: n_s = 2, ***N*** **= 15** , N_0 **= 6**, Q_c **= 9**, Chemical Formula: $C_{192}H_{248}N_{66}O_{120}P_{18}Ag_{15}$ and n_s = 2, ***N*** **= 12** , N_0 **= 4**, Q_c **= 8**, Chemical Formula: $C_{192}H_{248}N_{66}O_{120}P_{18}Ag_{12}$					
DNA-6		*Experimental*	**1890.8**	**0.101**	
	z = 4−	Q_c = 0	1893	0.00481	–2.2
	N **= 15**	Q_c = 8	1891	0.00481	–0.2
	N_0 **= 6**	Q_c **= 9**	**1890.7**	**0.00481**	**0.1**
		Q_c = 10	1890.5	0.00481	0.3

	z = 5– **N = 15** **N_0 = 6**	*Experimental*	**1512.4**	**0.0865**	
		$Q_c = 0$	1514.2	0.00384	–2.2
		$Q_c = 8$	1512.6	0.00384	–0.2
		Q_c = 9	**1512.4**	**0.00384**	**0.0**
		$Q_c = 10$	1512.2	0.00384	0.2
	z = 4– **N = 12** **N_0 = 4**	*Experimental*	**1810.1**	**0.107**	
		$Q_c = 0$	1812.1	0.00493	–2.0
		$Q_c = 7$	1810.3	0.00493	–0.2
		Q_c = 8	**1810.1**	**0.00493**	**0.0**
		$Q_c = 9$	1809.8	0.00493	0.3
	z = 5– **N = 12** **N_0 = 4**	*Experimental*	**1447.8**	**0.0849**	
		$Q_c = 0$	1449.4	0.00394	–2.4
		$Q_c = 7$	1448	0.00394	–0.2
		Q_c = 8	**1447.8**	**0.00394**	**0.0**
		$Q_c = 9$	1447.6	0.00394	0.2

References.

1. S. M. Copp, P. Bogdanov, M. Debord, A. Singh and E. Gwinn, *Adv. Mater.*, 2016, **28**, 3043.
2. S. M. Copp, A. Gorovits, S. M. Swasey, S. Gudibandi, P. Bogdanov and E. G. Gwinn, *ACS Nano*, 2018, **12**, 8240-8247.
3. S. M. Swasey, S. M. Copp, H. C. Nicholson, A. Gorovits, P. Bogdanov and E. G. Gwinn, *Nanoscale*, 2018, **10**, 19701-19705.
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